Remarks

Claims 1-11 are pending in this application. Claim 12-15 were previously cancelled. Claims 3 and 5 are currently withdrawn. Claim 1 has been amended to include the limitation that the interfering factor is a drug. This amendment finds support at page 2, second paragraph of the specification. Claim 1 also is amended to more clearly define which portion of the AT binding factor is being determined at each step of the claimed process. To the extent the claim is amended for clarity, those amendments are not intended to limit the scope of the claim.

Rejections Under 35 U.S.C. § 112, First Paragraph

The rejection of the claims under consideration for failing to comply with the written description requirement is respectfully traversed, because the specification describes the claimed subject matter in such a way as to reasonably convey to one skilled in the relevant art that the inventor, at the time the application was filed, had possession of the claimed invention.

The present invention is a method of determining AT in a sample that may contain an interfering factor. The invention lies in the use of an excess of AT binding partner, such that a portion of the AT binding partner interacts with the interfering factor under conditions in which the AT binding partner does not interact with AT so that the interfering factor no longer interferes with the subsequent determinations of the remaining free AT binding partner both before and after it has reacted with the AT, the difference between the two determinations being indicative of the amount of AT in the sample.

The Examiner has rejected the claims on the basis that the "claimed reagents" and "interfering factor" are not adequately described in the specification. At the outset, it should be noted that it is not the reagents themselves that are claimed, but the novel *sequence of steps* for using the reagents to determine AT. Any reagents that perform the stated functions, whether now known or to be discovered in the future, will be understood by those skilled in the art to be suitable for the claimed sequence of steps of the present invention. As stated in the MPEP, "The absence of definitions or details for well-established terms or procedures should not be the basis of a rejection under 35 U.S.C. 112, para. 1, for lack of adequate written description." In this case, as will be shown, such suitable reagents are well known in the art.

With regard to the reagent R1 which contains the AT binding partner, the Examiner has stated that, "Absent a disclosed correlation between structure and function, one skilled in the art would not envisage possession of the genus of R1 reagents based on the definition of the two species thrombin and factor Xa." But the applicant is not claiming all reagents R1, i.e., all binding partners of AT, per se. Rather, applicant is claiming the *use* of such a reagent R1 in a novel sequence of steps to perform an analysis of AT. The applicant does not have to be in possession of all possible AT binding partners in order to be in possession of the novel sequence of steps of the invention.

With respect to particular biological molecules, as explained by the Federal Circuit, "(1) examples are not necessary to support the adequacy of a written description; (2) the written description standard may be met ... even where actual reduction to practice of an invention is absent; and (3) there is no per se rule that an adequate written description of an invention that involves a biological macromolecule must contain a recitation of known structure." *Falkner v. Inglis*, 448 F.3d 1357, 1366, 79 USPQ2d 1001, 1007 (Fed. Cir. 2006). The MPEP further points out that additionally, unique cleavage by particular enzymes, isoelectric points of fragments, detailed restriction enzyme maps, a comparison of enzymatic activities, or antibody cross-reactivity may be sufficient to show possession of the claimed invention to one of skill in the art. See *Lockwood*, 107 F.3d 1565,1572, 41 USPQ2d 1961,1966 ("written description" requirement may be satisfied by using "such descriptive means as words, structures, figures, diagrams, formulas, etc., that fully set forth the claimed invention"). MPEP § 2163.II.A.E.a.

In this case, those skilled in the art of AT determinations would understand the scope of the reagents R1, R2, and R3 that would be suitable for use in the novel sequence of steps of the claimed invention. In addition to the art already of record in this case, applicant refers to the art submitted herewith, some of which is cited in the specification at paragraphs [0003] and [0004]. (Applicant expects to submit full copies of these references with a Supplemental Information Disclosure Statement in the near future; partial copies are submitted herewith for preliminary review.) Lill et al. discloses a detailed description of AT determination methods, including the various reagents that can be used at each step. Chromogenic substrates are discussed in the Fareed et al., and Abildgaard et al references. These references show that various reagents suitable for carrying out the novel claimed method were known in the art at the time of the invention. The particular structural characteristics of any of the reagents are not critical to the

ability of one skilled in the art to practice the inventive method of determining AT. Those skilled in the art, upon reading the disclosure of the present specification and the claimed sequence of steps, will be able to select known reagents that will perform the recited functions in the sequence of steps to be able to practice the claimed invention.

For example, with regard to reagent R2 which used is used to determine the binding partner of R1, applicant is not "attempting to describe an unknown by reference to another unknown," as the Examiner suggests. One skilled in the art, upon selecting an AT binding partner for reagent R1, will be sufficiently skilled to choose a reagent R2 that will allow the determination of that selected AT binding partner. Those skilled in the art similarly will know how to select reagents for R3, and an additional AT binding partner of claim 10.

Not everything necessary to practice the invention need be disclosed. In fact, what is well-known is best omitted. *In re Buchner*, 929 F.2d 660, 661, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991). All that is necessary is that one skilled in the art be able to practice the claimed invention, given the level of knowledge and skill in the art. See, e.g., *In re Fisher*, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970). MPEP 2164.08. In this case, one skilled in the art of AT determination will have sufficient knowledge to choose reagents to practice the sequence of steps that is the subject matter of the invention. Accordingly, the written description is adequate.

Rejections Under 35 U.S.C. § 112, Second Paragraph

The claims were rejected as indefinite under the second paragraph of 35 U.S.C. 112 because in claim 1 the recitation of "the free fraction of the AT binding partner" lacked antecedent basis, and because step (b) was believed to be indefinite. It is respectfully submitted that the present amendments to claim 1 correct these deficiencies, and it is respectfully requested that this ground of rejection be withdrawn.

Rejections Under 35 U.S.C. § 103

The rejection of claims 1-2, 4, 6-7, and 11 as obvious over Plattner et al. in view of Furatu, Morris et al, and Akhavan-Tafti et al. is respectfully traversed.

One aspect of the invention lies in the surprising discovery that it is possible to conduct two reliable determinations of free fractions of AT binding partner successively in one and the same sample. Yet, the skilled person upon reading Plattner et al., would have expected that a

reliable comparison between the first and second determinations of free fractions of AT binding partner would not have been possible, due to the addition of the R3 reagent between the first and second determinations.

As the Examiner correctly notes, Plattner et al. differs from the claimed invention in that it fails to specifically teach conducting the two measurements of the same substance at different times in a single reaction mixture. The Furatu reference does not teach the analysis of a single substance by two separate measurements in a single reaction mixture. Instead, Furatu in the paragraph spanning pages 3-4, teaches the analysis of a "first analysis item," then adding a reagent to measure a "second analysis item," the calculation of the concentration of the "second analysis item" being corrected to account for the amount of reagent added. This does not teach comparative analyses of any single analyte, and certainly not of an AT binding partner under different reaction conditions to determine AT. The Morris reference teaches repetitive tests on the same sample over a period of time to confirm the presence of a disease. Morris does not teach taking the difference between any two tests to determine a particular analyte. The Akhvan-Tafti reference also teaches taking the measurements of two different analytes at a single time, not using the difference between two measurements at different times and under different condition on the same sample to determine a single analyte.

The examiner's open-ended statement, "it would have been obvious to use known techniques to improve upon known methods in which multiple measurements are performed, such as those of Plattner et al." (Office Action page 10) does not state how the references would be combined. In fact, the references which teach different measurements of different analytes cannot be combined to give or render obvious the claimed sequence of steps used to measure AT. The Examiner states that these references teach the desirability of performing multiple measurements sequentially on a single sample, instead of in parallel on multiple samples (Office Action, p. 14). But none of them teach or suggest performing measurements of the same substance under different conditions and comparing the measurements in order to obtain a determination of an analyte, which is the essence of the present invention.

The Examiner's suggestion that one skilled in the art would have employed "creative steps" is belied by a presentation at the 2008 GTH Congress (Congress of Gesellschaft für Thrombose-und-Hämostaseforschung, held February 20-23, 2008, Wiesbaden, Germany), a meeting of those skilled in the art. The Hickey monograph shows that the problem of the

presence of the drug Leprudin as an interfering factor in determinations of AT still had not been solved. If employing the "creative step" of the present invention would have been obvious to those skilled in the art, those steps would have been so employed by the time of the GTH 2008 congress. The "creative step" that the Examiner dismisses as obvious is in fact one that has not yet been employed by others to solve this long-recognized need in the art.

As claim 1 is non-obvious, the remaining claims dependent from claim 1 also are non-obvious.

In view of the foregoing, a notice of allowance is respectfully requested.

McDonnell Boehnen Hulbert & Berghoff LLP

/Patrick G. Gattari/
Patrick G. Gattari
Reg. No.: 39,682

McDonnell Boehnen Hulbert & Berghoff
300 South Wacker Drive
Chicago, Illinois 60606
Tel: 312 913 0001
Fax: 312 913 0002

3.3 Plasma Proteinase Inhibitors

3.3.1 Antithrombin III

Heparin Cofactor

Helmut Lill and Peter Röschlau

General

Early in this century it was recognized that thrombin progressively loses activity when incubated with defibrinated plasma. It was assumed that an inhibitor of thrombin must be responsible for the observed inactivation. Later, as the anticoagulant effect of heparin in blood was discovered, it could be demonstrated that heparin exhibits its action only in the presence of a component of the blood plasma, the heparin cofactor. For many years, the progressive thrombin inhibitor (antithrombin III, AT III) and the heparin cofactor were regarded as different molecules.

However, in 1968 evidence was provided by *Abildgaard* [1], by isolation and characterization of the pure protein, that both inhibitory activities belong to one and the same molecule. These observations were further substantiated by the work of *Yin et al.* [2] and *Rosenberg & Damus* [3].

Antithrombin III (AT III) must be considered the main physiological inhibitor of blood coagulation. A decreased AT III concentration in plasma results in recurrent thrombo-embolic complications [4]. AT III is a single-chain glycoprotein (M_r 62000 – 67000) which is synthesized in the liver. The normal range of concentration in human plasma has been estimated to be 240 ± 33 mg/l [5]. AT III inhibits most of the known serine proteases of the coagulation system. Inhibitory reactions against plasmin, kallikrein, trypsin and chymotrypsin have been described. Inhibition involves the formation of a 1 : 1 molecular complex of enzyme and inhibitor. The rate of complex formation, especially with thrombin and Factor Xa, is greatly increased in the presence of heparin [2, 3].

Application of method: in clinical chemistry and in biochemistry.

Inhibitor properties relevant in analysis: different AT III patterns were observed in families with congenital AT III deficiency. The behaviour of such AT III molecules varies with respect to immunological and functional assays, electrophoretic mobility and heparin binding affinity [6, 7].

Methods of determination: the determination of AT III has been described using immunological assays (*Mancini* or *Laurell* technique, laser-nephelometric quantitation [5], radioimmunoassay [8]) or functional assays [9 – 11]. The findings of *Sas et*

al. [6] and *Tran et al.* [7] indicate that the functional assay must be considered to be more relevant for clinical purposes.

The functional methods can be divided into clotting assays using fibrinogen as a substrate and assays with synthetic substrates. Both types of assays can be performed with either thrombin or factor Xa, with or without the use of heparin.

The use of heparin shortens the incubation time considerably, thereby increasing the specificity of the assay. Moreover, heparin present in the sample does not interfere. With the present state of knowledge, synthetic substrate assays must be considered superior to clotting assays with respect to precision and standardization [12].

4-Nitroanilides of tri- or tetrapeptides are most widely employed as substrates, but peptides containing fluorigenic groups [13] or thioesters [14] are also used.

Active-site titration has been proposed as a means of thrombin standardization or calculation of AT III concentration on a molar basis [3, 9].

The method presented here is a well-proved functional assay procedure using thrombin, the 4-nitroanilide substrate Chromozym® TH, and heparin [15].

International reference method and standards: no international reference method exists at present.

An international reference preparation for human AT III is available from the National Institute for Biological Standards and Control, Holly Hill, Hampstead, London.

Effectors of the AT III reaction: inactivation of thrombin or Factor Xa by AT III is greatly accelerated by heparin. Heparin acts as a catalyst during the inactivation reaction. Several years ago *Rosenberg & Damus* [3] assumed that this effect must be due to an allosteric change in the AT III molecule after heparin binding.

Now evidence has strengthened that linkage of both AT III and thrombin to heparin is required for a rapid inactivation of thrombin by AT III (approximation [16] or template model [17]).

However, it is interesting to note that, for the rapid inactivation of Factor Xa, there seems to be no need for a direct Factor Xa-heparin interaction. In this case the heparin-induced allosteric change in AT III is considered to be the sole principle of inhibitory acceleration [16].

Assay

Method Design

Principle

(a) $\text{AT III} + \text{heparin} \longrightarrow \text{AT III} \cdot \text{heparin}$

(b) $\text{AT III} \cdot \text{heparin} + \text{thrombin (excess)} \longrightarrow \text{AT III} \cdot \text{heparin} \cdot \text{thrombin} + \text{thrombin (residual)}$

(c) Tos-Gly-Pro-Arg-4-NA + H_2O $\xrightarrow{\text{thrombin (residual)}}$ Tos-Gly-Pro-Arg-OH + 4-nitroaniline .

AT III is incubated in buffered solution with heparin and thrombin. After formation of the AT III-thrombin complex the thrombin substrate Tos-Gly-Pro-Arg-4-NA (Chromozym® TH) is added and residual thrombin activity is measured at 405 nm. Residual thrombin activity is inversely proportional to the AT III concentration of the sample. The difference between the thrombin blank reaction and the sample reaction is used for calculation of AT III activity in international units.

The method described below can easily be adapted to automated devices [15].

Optimized conditions for measurement: a fast and quantitative AT III-thrombin complex formation can be obtained by using both heparin and thrombin in sufficient excess. This renders the assay relatively independent of incubation time (complex formation is completed within 1 – 2 min) or of variations in thrombin activity used (± 20%). In the range from 7.0 to 9.0 the pH of the incubation mixture is optimal for complex formation: pH 8.1 provides reliable conditions for measurement of thrombin activity and AT III *vice versa*.

Temperature conversion factors: factors determined in our laboratory for relating measurements at 30 °C to values at 25 °C, or 37 °C to 25 °C, are 1.35 and 1.89, respectively.

Equipment

Spectrophotometer or spectral-line photometer capable of exact measurements at 405 nm (400 – 420 nm), preferably with a thermostatted cuvette holder; water-bath; recorder or stopwatch.

Reagents and Solutions

Purity of reagents: the thrombin preparation must be free from Factor Xa. Suitable commercial preparations are available. The purity can be checked using soybean trypsin inhibitor, 50 µg/ml, with the thrombin solution.

Preparation of solutions (for about 50 determinations): all solutions in re-purified water (cf. Vol. II, chapter 2.1.3.2).

1. Buffer/heparin solution (Tris, 0.1 mol/l, pH 8.1; heparin, 2.0 USP units/ml; aprotinin, 10 µg/ml; sodium chloride, 0.15 mol/l; EDTA, 0.01 mol/l; PEG* 6000, 1% (w/v)):

* Polyethylene glycol.

dissolve 1.21 g Tris + 0.88 g NaCl + 0.37 g EDTA-$Na_2H_2 \cdot 2\ H_2O$ + 1.0 g PEG 6000 in about 70 ml water, add 1.0 mg aprotinin + 200 USP units heparin, adjust to pH 8.1 with HCl, 2 mol/l, and dilute to 100 ml final volume with water.

2. Thrombin solution (500 IU*/l, 25°C, pH 8.1, in buffer solution 1):

   dissolve 2.5 U bovine thrombin in 5 ml solution (1). It is important to use a plastic bottle or siliconized glassware because thrombin is adsorbed on glass.

3. Substrate solution (Tos-Gly-Pro-Arg-4-Na · acetate, Chromozym® TH, 1.875 mmol/l; glycine, 66.6 mmol/l):

   dissolve 12.42 mg Chromozym® TH + 50 mg glycine in 10 ml water.

4. Reagent (Tris, 0.1 mol/l; heparin, 2.0 USP units/ml; NaCl, 0.15 mol/l; EDTA, 10 mmol/l; PEG 6000, 0.9% w/v; thrombin, 24 U/l; aprotinin, 10 µg/ml):

   mix in a plastic bottle 1 part of solution (2) with 20 parts of solution (1) (e.g. for 10 assays mix 1 ml of solution (2) + 20 ml of solution (1)). It is important to use plastic pipettes for solution (2) and a plastic bottle for solution (4) because thrombin is adsorbed on glass. Let solution (4) stand prior to use for about 30 min to assure an equilibrium of thrombin activity.

Stability of solutions: store all solutions at 0–4°C. Solution 1 (buffer/heparin) is stable as long as no bacterial or fungal contamination occurs. Thrombin solution (2) is stable for 2 weeks. Substrate solution (3) is stable for at least 4 weeks if contamination is excluded. It is advisable to prepare reagent solution (4) freshly before use. Solution (4) is stable for at least 8 h at 15° to 25°C.

Procedure

Collection and treatment of specimen: collect blood from the vein without stasis. Mix 9 parts by volume of blood during collection into 1 part by volume of sodium citrate solution, 0.11 mol/l. The specimen must be centrifuged at about 2000 *g* (about 3000 rpm on conventional laboratory centrifuges) for 10 min within 2 h of collection. Before assay 1 part by volume of plasma is diluted with 50 parts by volume of NaCl solution, 0.9% (w/v) (e.g. 20 µl plasma + 1 ml NaCl solution).

Stability of AT III in the sample: citrated plasma may be stored up to 48 h at +4°C or 6 months at −20°C. Frozen plasma must be thawed at 25°C about 15 min prior to the assay and mixed gently before use. Diluted plasma is stable for at least 8 h at +4°C.

* International units of thrombin activity; Chromozym® TH as substrate.

Details for measurement in other specimens: in principle, measurement in serum is possible but, for clinical purposes, it is not relevant due to a reduced AT III concentration. If using serum, care should be taken in respect of a sample blank reaction. With plasma, other anticoagulants (oxalate, 1 mg/ml; heparin, 0.2 mg/ml; EDTA, 1 mg/ml) can also be used. However, the different ratios of blood to anticoagulant used will influence the test result.

Assay conditions: wavelength Hg 405 nm; plastic cuvette; light path 10 mm; final volume 2.30 ml; temperature* 25 °C or 30 °C (thermostatted cuvette holder). Measure against air. Before starting the assay adjust all solutions to assay temperature. Thrombin is adsorbed on glass. Accordingly, only plastic pipettes may be used to pipette reagent solution (4). At least one thrombin blank is required per assay series.

Measurement

Pipette successively into plastic cuvettes:	blank	sample	concentration in assay mixture
dil. plasma (or other sample)	–	0.10 ml	volume fraction (undiluted sample) 0.0008
NaCl solution 0.9%	0.10 ml	–	
reagent (4)	2.00 ml	2.00 ml	thrombin 20.9 U/l heparin 1.74 USP units/ml Tris 87 mmol/l NaCl 0.131 mol/l EDTA 8.7 mmol/l aprotinin 8.7 mg/l PEG 6000 0.87% (w/v)
mix thoroughly with a plastic spatula, incubate for 5 min at assay temperature (25 °C or 30 °C),			
substrate (3)	0.20 ml	0.20 ml	substrate 0.16 mmol/l
mix immediately, read absorbance within 30 s and start stopwatch at the same time. Repeat reading after exactly 30, 60 and 90 s or monitor the reaction on a recorder.			

If $\Delta A_{AT\,III}/\Delta t$ for 30 s is less than 0.012 it is recommended to repeat the assay using plasma (sample) diluted with only half as much NaCl solution (result × 0.51).

* For measurement at 37 °C dilute 1 part of sample with 100 parts of NaCl solution (e.g. 20 µl sample + 2 ml NaCl solution). Prepare reagent solution (4) with half the amount of thrombin solution (2).

Calculation: 1 unit is the amount of AT III which inactivates 1 international unit of thrombin. Subtract the mean absorbance change per 30 s of the sample reaction from the thrombin blank, multiply by 2 and use this $\Delta A/\Delta t$ for calculation. Use calculation formula (k) or (k_1), cf. "Formulae", Appendix 3. Measuring at Hg 405 nm, $\varepsilon = 0.97$ $l \times mmol^{-1} \times mm^{-1}$, the following relation is valid for the catalytic concentration of AT III in the sample (t in minutes):

$$b = \frac{1000 \times 2.3}{0.97 \times 10 \times 0.1} \times F \times \Delta A/\Delta t = 2371 \times F \times \Delta A/\Delta t \quad U/l$$

$$b = 39519 \times F \times \Delta A/\Delta t \quad nkat/l$$

F is sample dilution factor (51 in this assay).

For measurement at wavelengths other than 405 nm an AT III standard must be used for calculation.

For measurement at 37°C use sample dilution factor F = 101.

Validation of Method

Precision, accuracy, detection limit and sensitivity: we determined the within-run imprecision of the assay (25°) using plasma samples containing AT III levels of 12400, 6900, and 2500 U/l. The standard deviations (s) were 140, 140, and 170 U/l. The relative standard deviations referred to the mean value of a series (n = 25) were 1.1%, 2.1%, and 7.0%.

In an interlaboratory trial [12] samples containing 4450 and 9680 U/l were assayed, the relative standard deviations being 6.9% and 6.5% respectively. To study the inaccuracy of the assay, recovery experiments with isolated AT III were performed in plasma. The percentage of theoretical recovery was always better than 93% [15]. The detection limit of the assay is approximately an AT III concentration of 1000 U/l, a value measurable with an imprecision of ± 30%.

Taking into account the imprecision of the method, an AT III difference between two samples of 1000 U/l ($\Delta A_{AT\,III}/\Delta t = 0.004$ (30 s) can be determined.

Sources of error: haemoglobin or bilirubin in plasma do not interfere with the assay. Heparin-neutralizing agents may influence the assay by decreasing the heparin concentration <1.30 USP units/ml.

Specificity: until recently, the heparin-enhanced AT III assay could be considered absolutely specific. However, *Wunderwald et al.* [18], *Friberger et al.* [19] and *Tollefsen et al.* [20] have described a new heparin-dependent thrombin inhibitor (AT-BM or second heparin cofactor) in plasma. This new inhibitor exhibits a higher affinity for

human thrombin than for bovine thrombin. Moreover, the rate of thrombin inhibition by this inhibitor is also relatively slow in the presence of high heparin concentrations. High plasma dilution, as used in the AT III assay, further minimizes interference by the newly-discovered inhibitor.

The specificity of the AT III assay which uses bovine thrombin, short incubation time and high plasma dilution was confirmed by an inter-laboratory clinical trial [21].

Reference ranges: *Hesse et al.* [21] found in plasma from normal adults AT III activities ranging from 10000 to 15000 U/l at 25 °C and from 20000 to 29000 U/l at 37 °C.

References

[1] *U. Abildgaard,* Highly Purified Antithrombin III with Heparin Cofactor Activity Prepared by Disc Electrophoresis, Scand. J. Clin. Lab. Invest. *21*, 89–91 (1968).

[2] *E. T. Yin, S. Wessler, P. J. Stoll,* Identity of Plasma-Activated Factor X Inhibitor with Antithrombin III and Heparin Cofactor, J. Biol. Chem. *246*, 3712–3719 (1971).

[3] *R. D. Rosenberg, P. S. Damus,* The Purification and Mechanism of Action of Human Antithrombin-Heparin Cofactor, J. Biol. Chem. *248*, 6490–6505 (1973).

[4] *O. Egeberg,* Inherited Antithrombin Deficiency Causing Thrombophilia, Thromb. Diath. Haemorrh. *13*, 516–530 (1965).

[5] *Z. Parvez, J. Fareed, H. L. Messmore, R. Moncada,* Laser Nephelometric Quantitation of Antithrombin III (AT III). Development of a New Assay, Thromb. Res. *24*, 367–377 (1981).

[6] *G. Sas, G. Blasko, J. Bonhegyi, J. Jako, A. Palos,* Abnormal Antithrombin III (Antithrombin "Budapest") as a Cause of Familial Thrombophilia, Thromb. Diath. Haemorrh. *32*, 105–115 (1974).

[7] *T. H. Tran, H. Bounameaux, C. Bondeli, H. Honkanen, G. A. Marbet, F. Duckert,* Purification and Partial Characterization of a Hereditary Abnormal Antithrombin III Fraction of a Patient with Recurrent Thrombophlebitis, Thromb. Haemostasis *44*, 87–91 (1980).

[8] *V. Chan, T. K. Chan, V. Wong, S. C. Tso, D. Todd,* The Determination of Antithrombin III by Radioimmunoassay and its Clinical Application, Br. J. Haematol. *41*, 563–572 (1979).

[9] *O. R. Ødegård, U. Abildgaard,* Antithrombin III: Critical Review of Assay Methods, Haemostasis *7*, 127–134 (1978).

[10] *H. Bounameaux, F. Duckert, M. Walter, J. Bounameaux,* The Determination of Antithrombin III. Comparison of Six Methods. Effect of Oral Contraceptive Therpay, Thromb. Haemostasis *39*, 607–615 (1978).

[11] *R. D. Philo, P. J. Gaffney,* Comparison of Antithrombin III Assays Using Biological and Chromogenic Substrates, Br. J. Haematol. *50*, 147–156 (1982).

[12] *I. Witt,* Ergebnisse eines "Ringversuchs" für Antithrombin III, in: *J. v. de Loo, F. Asbeck* (eds.), Hämostase, Thrombophilie und Arteriosklerose, F. K. Schattauer Verlag, Stuttgart, New York 1982, pp. 608–614.

[13] *R. C. Bishop, P. M. Hudson, G. A. Mitchell, S. P. Pochron,* Use of Fluorogenic Substrates for The Assay of Antithrombin III and Heparin, Ann. N. Y. Acad. Sci. *370*, 720–730 (1981).

[14] *G. D. J. Green, E. Shaw,* Thiobenzyl Benzyloxycarbonyl-L-Lysinate, Substrate for a Sensitive Colorimetric Assay for Trypsin-Like Enzymes, Anal. Biochem. *93*, 223–226 (1979).

[15] *K. Bartl, H. Lill,* A Versatile Assay Method of Antithrombin III Using Thrombin and Tos-Gly-Pro-Arg-pNa, Thromb. Res. *18*, 267–272 (1980).

[16] *R. D. Rosenberg,* The Function and Structure of the Heparin Molecule, in: *I. Witt* (ed.), Heparin, New Biochemical and Medical Aspects, Walter de Gruyter, Berlin, New York 1983, pp. 3–27.

[17] *M. J. Griffith,* Kinetics of the Heparin-Enhanced Antithrombin III/Thrombin Reaction. Evidence for a Template Model for the Mechanism of Action of Heparin, J. Biol. Chem. *257*, 7360–7365 (1982).

[18] *P. Wunderwald, W. J. Schrenk, H. Port,* Antithrombin BM from Human Plasma: An Antithrombin Binding Moderately to Heparin, Thromb. Res. *25*, 177–191 (1982).
[19] *P. Friberger, N. Egberg, E. Holmer, M. Hellgren, M. Blombäck,* Antithrombin Assay – the Use of Human or Bovine Thrombin and the Observation of a "Second" Heparin Cofactor, Thromb. Res. *25*, 433–436 (1982).
[20] *D. M. Tollefsen, D. W. Majerus, M. K. Blank,* Heparin Cofactor II: Purification and Properties of a Heparin-Dependent Inhibitor of Thrombin in Human Plasma, J. Biol. Chem. *257*, 2162–2169 (1982).
[21] *L. Roka, I. Witt, T. H. Schöndorf, G. Oehler,* unpublished results (1983).
[22] *R. Hesse, W. Tritschler, G. Castelfranchi, W. Bablok,* Antithrombin III: Referenzwerte mit einem chromogenen Substrat (Chromozym® TH), Blut *42*, 227–234 (1981).

3.3.2 α_1-Proteinase Inhibitor (α_1-Antitrypsin)

Irene Witt and Helmut Lill

General

α_1-Antitrypsin constitutes the major serine proteinase inhibitor in human plasma and in the lower respiratory tract. It was first described by *Schultze et al.* [1] in 1962.

In the early 1970's it became apparent that this protein is not a specific inhibitor of trypsin, but is a relatively unspecific inhibitor of proteinases. It inhibits not only trypsin and chymotrypsin but also elastases from various sources (leukocytes, pancreas, skin), plasmin and thrombin. Therefore, the term α_1-proteinase inhibitor (α_1-Pi)* was suggested by *Pannell et al.* [2].

The most important physiological function of α_1-Pi is considered to be inhibition of elastase from leukocytes.

Although the mechanism of inhibition has not been definitely elucidated, a 1 : 1 molar complex between α_1-Pi and proteinase is formed during inhibition.

The plasma form of the inhibitor is synthesized by the liver. The purified protein has a molecular weight of approx. 53000. It is a glycoprotein with a total carbohydrate content between 11 and 18%. The carbohydrate moiety consists of *N*-acetyl glucosamine, mannose, galactose and *N*-acetyl neuraminic acid [3, 4].

An interesting feature of the α_1-proteinase inhibitor is its genetic polymorphism, which was first demonstrated by showing that α_1-Pi from deficient individuals has a different mobility on agarose electrophoresis [5]. It is possible to recognize gene products from more than 40 alleles by isoelectric focusing, which are alphabetically

* Abbreviations: α_1-Pi α_1-Proteinase inhibitor
BAPA Benzoylarginine 4-nitroanilide
4-NA 4-nitroaniline
PEG Polyethylene glycol.

18. Current status of methodologies for antithrombin III and heparin with the advent of peptide chromogenic substrates

J. FAREED, H. L. MESSMORE and J. U. BALIS

Introduction

Since the emergence of synthetic peptide chromogenic substrates clinical and research laboratory methodologies for the assessment of hemostatic disorders have undergone a significant conceptual change which views coagulation process as a dynamic equilibrium between active enzymes and inhibitors. Chromogenic substrate methods have been developed for the control and management of heparin and oral anticoagulant therapy. Specific chromogenic substrates for thrombin have been employed to assay prothrombin, thrombin-like enzymes, and the natural and synthetic inhibitors of thrombin. Of these anticoagulant activities antithrombin III and heparin have been studied most extensively.

AT-III Assay by chromogenic substrates

In our laboratory we have developed simple methods for the estimation of AtIII activity.

Test material

Normally the AtIII determinations are made on citrated plasma obtained by using a double syringe method in order to minimize the activation of coagulation enzymes.

A normal human pool plasma (NHP) should be prepared in identical manner as the test sample; preferably the blood should be drawn from 20 normal, healthy volunteers, 18-45 years old. Once the pool is prepared we generally assay it against a standard AtIII preparation which is available from the American National Red Cross, Washington D.C., or A.B. KABI, Molndal, Sweden. Aliquots of this plasma can be kept in plastic tubes at -70°C for up to 6 months without any significant loss of AtIII.

Thrombin

Our recommendation is to use either the Fibrindex^{R} or human thrombin (10 U/ml) frozen at -70°C in aliquots of 1 ml. The amidolytic as well as the clotting activity of this thrombin preparation is stable under these conditions (Table 18.1).

Our assay method requires a small amount of plasma (2-5 µl) or 25-50 µl of a 1:10 dilution of plasma which is mixed with 0.85 ml of buffer (pH 9.2, 0.2 M Tris HCl containing 3 µ/ml heparin). A known amount of thrombin (50 µl of 10 µ/ml) is then mixed with the mixture and incubated for one minute at room temperature. After this time 10.0 µl of a 2:1 mixture of S-2238 (0.75 mg/ml and polybrene (1 mg/ml) is added and the optical density change is recorded.

2.6 Studies on Antithrombin III Using Chromozym TH

H. Stormoken

We have adapted the system of Ødegaard et al. (1) for the assay of antithrombin-III using Chromozym TH (Pentapharm, Basle) as substrate for amidolysis. The system used was as illustrated in table 1.

Table 1

3.9 ml glycin/NaOH, 0.1 M, pH 8.4 with 3 IU heparin/ml

0.1 ml plasma/serum

→400 µl (= 10 µl plasma)

100 µl thrombin, 7.5 U/ml

30" incub.

300 µl 0.33 mM C-TH with 0.4 mg polybrene/ml

60" incub.

300 µl conc. acetic acid

The standard dilution curve of a normal plasma pool (25) was rectilinear between 140 and 40 % of the normal pool (15-4 µl plasma). The correlation with the electroimmuno method using AT-III antibody from Nyegaard & Co. (Oslo) was r = 0.84.

Using this modification we investigated the AT-III level in 222 persons belonging to a clinical prospective material under investigation for coronary disease. The material is investigated for a large series of parameters, including the

THROMBOSIS RESEARCH
Printed in Great Britain

Vol. 11, pp. 549-553, 1977
Pergamon Press, Ltd.

B R I E F C O M M U N I C A T I O N

ANTITHROMBIN (HEPARIN COFACTOR) ASSAY WITH "NEW" CHROMOGENIC SUBSTRATES (S-2238 AND CHROMOZYM TH)

U. Abildgaard, M. Lie & O.R. Ødegård

Medical Department A, Aker Hospital, Oslo 5, Norway

(Received 18.6.1977; in revised form 18.8.1977.
Accepted by Editor H.C. Godal)

INTRODUCTION

A two-stage method for the determination of antithrombin III (heparin cofactor) activity using the chromogenic substrate Bz-Phe-Val-Arg-pNA (S-2160) was recently described (1). The relative simplicity of the method and the close correlation to results of immunoassay makes the method well suited for routine work. In this laboratory it has been used for assay of antithrombin III (At-III) in about 3000 plasma samples. S-2160 is somewhat difficult to dissolve. Two new thrombin substrates, H-D-Phe-Pip-Arg-pNA (S-2238) (2) and Tos-Gly-Pro-Arg-pNA (Chromozym TH) (3) are readily soluble, and may be amidolyzed by thrombin at rates two to three times higher than observed for S-2160 (2). These substrates have enabled us to improve the assay. The amidolytic reaction time is shortened, and plasma is examined at a higher dilution.

METHODS

Test plasma. Blood (9 vol) is mixed with 0.1 M sodium citrate (1 vol) and centrifuged (2000 g, 20 min). Plasma is pipetted off and kept at about 4 °C or stored at -20 °C until assay.

Reference plasma. Equal amounts of citrated plasma from ten healthy individuals are pooled. Aliquots are stored at -20 °C.

Buffer with heparin. 250 ml Tris (0.2 M), 20 ml HCl (1M), 30 ml K_2EDTA (0.25 M), 0.6 ml heparin (5000 U/ml), final volume adjusted to 1000 ml with 0.26 M NaCl. pH 8.4, ionic strength 0.2, heparin concentration 3.0 U/ml (Porcine mucosa heparin from AL, Oslo, Norway, lot no 42456).

Thrombin. Bovine (Topostasine, Roche, Basle, Switzerland) 3000 NIH U/vial, lot no B 112092 T, and Human (Fibrindex, Ortho, New Jersey, USA) 50 NIH U/vial, lot no 5P223, were dissolved in NaCl (0.15 M) to 100 or 50 NIH U/ml and stored at -20 °C (maximum storage time about 3 months). Working solutions (about 10-15 NIH U/ml) were kept on ice and used within one hour.

Substrates. H-D-Phe-Pip-Arg-pNA (S-2238) from Kabi, Stockholm, Sweden, and Tos-Gly-Pro-Arg-pNA (Chromozym-TH) from Pentapharm, Basle, Switzerland, were dissolved in distilled water to 1.5 mmol/l and stored in the dark at 4 °C. Stability above 6 months.

Substrate-polybrene solutions. One vol. substrate solution (1.5 mmol/l) is mixed with one vol. polybrene (0.6 mg/ml in distilled water) (Polybrene from Abbott, Chicago, USA) (polybrene may be omitted, Cf. Results).

At-III immunoassay was performed with a single radial diffusion method (4), using antiserum from Nyegaard, Oslo, Norway.

Statistical methods. The correlation coefficient (r) and the linear regression of Y on X were calculated with the Compucorp 141 calculator (Los Angeles, USA).

Assay procedure: Test plasma is diluted one in sixty in buffer with heparin (e.g. 100 µl plasma + 5.9 ml buffer). 400 µl of this dilution is prewarmed at 37 °C.

Stage I is started by the addition of 100 µl thrombin solution (10-15 NIH U/ml, Cf. Results) into the test tube and starting a stop watch. After 30 sec, stage II is initiated by adding 300 µl substrate-polybrene solution. Exactly 30 sec after addition of the substrate-polybrene solution, stage II is terminated by the addition of 300 µl concentrated acetic acid into the mixture. Complete mixing is essential.

Absorbance is read at 405 nm against a blank containing 400 µl reference plasma dilution, 300 µl acetic acid and 400 µl distilled water.

Standard curve: Reference plasma diluted 1+59 is defined as 100 per cent activity. A full standard curve is obtained using dilutions in the 1/240 - 1/50 range (25-120 %, see Fig.1). For routine work, only the 25 and 120 % values are needed.

RESULTS

Linear relations between absorbance and plasma concentration were obtained for both the two chromogenic substrates and both thrombin preparations. The thrombin concentration should be adjusted to yield an absorbance value of about 0.85 (Fig.1). At low thrombin concentrations, the standard curve deviated from linearity at the highest plasma concentrations, apparently due to inhibitor excess. At high thrombin concentrations, the discriminating sensitivity is reduced.

Standard curves obtained in the absence and presence of polybrene (stage II) were identical. Polybrene may therefore be omitted. The present results were obtained with polybrene.

The incubation time in stage I is not critical. At-III values obtained with either 30 or 120 sec incubation differed by 5 per cent or less. In contrast, the amidolysis time (stage II) must be kept as uniform as possible.

A close correlation was found between the At-III activity and the results of immunoassay (Fig.2). The standard deviation was 2.9 per cent when calculated from duplicate determinations in 30 plasma samples (mean activity 84, range 40-25 per cent).

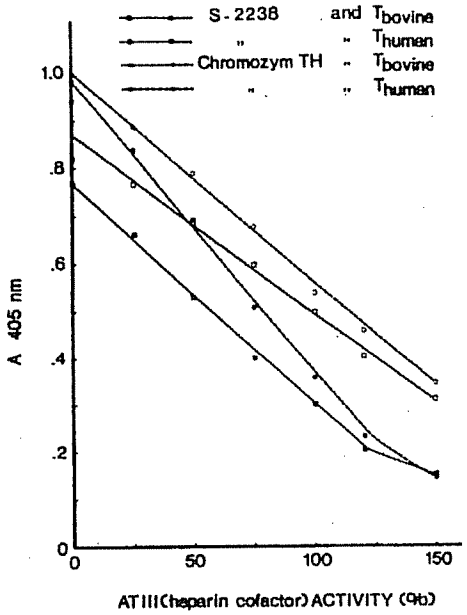


Fig.1. Reference curves for At-III activity assays in plasma. Reference plasma diluted 1/60 is defined as 100 per cent activity.(Thrombin:15 NIH U/ml).

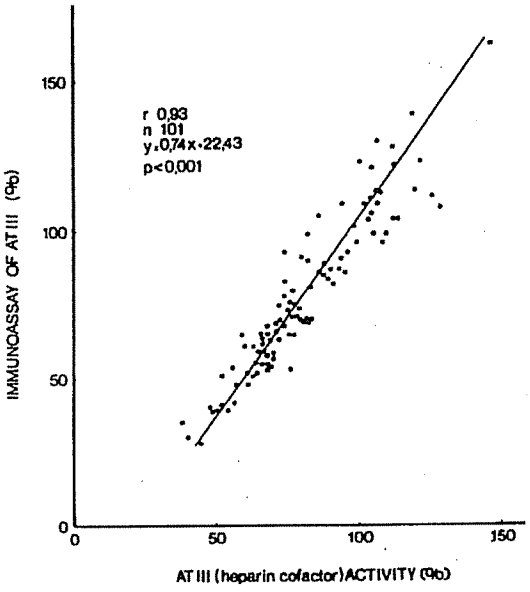


Fig.2. Correlation between At-III activity (S-2238, bovine thrombin) and immunoassay of At-III.

DISCUSSION

The chromogenic substrates have proved valuable for the study of inhibitors of coagulation (1,5-11). Assay methods reflecting the activity of At-III both in the absence (5,6,7,9) and presence of heparin (1,7,8,10) have been developed. With the exception of a Xa method performed in the absence of heparin (7), the results of these methods are closely correlated to the results of immunoassay. As practical considerations then may determine the choice of method, we have found the rapid methods (with heparin present during stage I) convenient. In the presence of heparin, virtually all the At-III in plasma reacts with thrombin, and the "heparin cofactor" assay measured the total thrombin inactivating capacity of diluted plasma. Our original "heparin cofactor assay" may be performed with the "new" substrates (10). We found it preferable, however, to modify the method as a higher dilution of plasma reduces errors due to bilirubin and turbidity. Individual plasma blanks are therefore only needed when bilirubin is above 8 mg/ml, or with grossly lipemic plasma. Other advantages are the higher solubility of the substrates and the shorter reaction times. The source of heparin used in the buffer is probably not important (11). In this report, we obtained similar results with a bovine and a human thrombin preparation. With some other thrombin preparations, the standard curves may be inadequate (11). This may reflect the presence of denatured thrombin which has amidolytic but not proteolytic activity (12).

REFERENCES

1. ØDEGÅRD,O.R.,LIE,M. and ABILDGAARD,U.: Heparin cofactor activity measured with an amidolytic method. Thromb.Res.6,287,1975.
2. CLAESON,G.,AURELL,L.,KARLSSON,G. and FRIBERGER,P.: Substrate structure and activity relationship. p.37 in New methods for the analysis of coagulation using chromogenic substrates. I. Witt,ed. de Gruyter,Berlin,New York 1977.
3. SVENDSEN,L. and STOCKER,K.: Die Bestimmung von Protease-Aktivitäten mit gezielt konstruirten Peptid-p-Nitroanilid-Derivaten. p.23 in New methods for the analysis of coagulation using chromogenic substrates. I.Witt,ed. de Gruyter,Berlin,New York 1977.
4. FAGERHOL,M.K. and ABILDGAARD,U.: Immunological studies on human antithrombin III. Scand.J.Haemat.7,10,1970.
5. BLOMBACK,M.,BLOMBACK,B.,OLSSON,P. and SVENDSEN,L.: The assay of antithrombin using a synthetic chromogenic substrate for thrombin. Thromb.Res.5,621,1974.
6. ABILDGAARD,U.,LIE,M. and ØDEGÅRD,O.R.: A simple amidolytic method for the determination of functionally active antithrombin III. Scand.J.Clin.Lab.Invest.36,109,1976.
7. ØDEGÅRD,O.R.,LIE,M. and ABILDGAARD,U.: Antifactor Xa measured with amidolytic methods. Haemostasis,5,265,1976.
8. STORMORKEN,H.: Studies on antithrombin III using chromozym TH. p.119 in New methods for the analysis of coagulation using chromogenic substrates. I.Witt,ed. de Gruyter,Berlin,New York 1977.

9. VINAZZER,H.: Assay of antithrombin III with the chromogenic substrate S-2160. p.61 in New Methods for the analysis of coagulation using chromogenic substrates. I.Witt, ed. de Gruyter, Berlin,New York,1977.

10. ØDEGÅRD,O.R. and ABILDGAARD,U.: Determination of antithrombin III and antifactor Xa activity. p.123 in New Methods for the analysis of coagulation using chromogenic substrates. I.Witt, ed. de Gruyter,Berlin,New York,1977.

11. ØDEGÅRD,O.R.: Evaluation of an amidolytic heparin cofactor assay method. Thromb.Res.7,351,1975.

12. GAFFNEY,P.J., LORD,K.,BRASHER,M. and KIRKWOOD,T.B.L.: Problems in the assay of thrombin using synthetic peptides as substrates. Thromb.Res.10,549,1977.

Clinical evaluation of a new FXa-based Antithrombin assay on Sysmex® CA-1500 System

Kieron Hickey[a], Peter Cooper[a], Steve Kitchen[a], Mechthild Merz[b] and Martina Böhm-Weigert[b]

[a] Sheffield Haemophilia and Thrombosis Centre, Royal Hallamshire Hospital, Sheffield, United Kingdom
[b] Dade Behring Marburg GmbH – A Siemens Company, Marburg, Germany

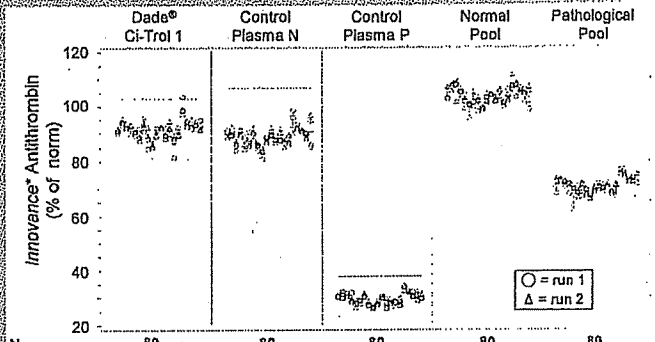
Objectives:

Screening for Antithrombin (AT) deficiency is done with FIIa- and FXa-based methods. FXa-based methods are often believed to be superior to FIIa-based methods because of the absence of Heparin Cofactor II interference. On the other hand FIIa-based assays have demonstrated in some patients higher sensitivity for certain AT type II defects[2,3]. In this study, a new FXa-based AT assay (*Innovance*® Antithrombin – Product under development, not available for sale) from Dade Behring Marburg GmbH - A Siemens Company, Marburg, Germany was evaluated and compared to the established FIIa-based Berichrom® Antithrombin III (A) assay from the same company.

Results and Discussion:

1. Precision of *Innovance*® Antithrombin

As depicted in Figure 1, the precision of the new assay is excellent, showing for controls and plasma pools within-device CVs (total CVs), repeatability CVs (within-run CVs) and between-run CVs between 3.0-6.4 %, 1.4-2.9 % and 0.4-2.4 %, respectively.



	Dade® Ci-Trol 1	Control Plasma N	Control Plasma P	Normal Pool	Pathological Pool
N	80	80	80	80	80
Mean	91.7	89.0	29.6	103.7	70.8
Repeatability	1.8	1.4	2.9	1.7	1.7
Within-day CV	2.1	2.7	3.0	2.2	2.9
Between-run CV	1.1	2.3	0.4	1.3	2.4
Between-day CV	3.0	2.3	5.7	2.1	3.0
Within-device CV	3.6	3.6	6.4	3.0	4.2

Figure 1: Precision data for *Innovance*® Antithrombin

2. Method Comparison between *Innovance*® Antithrombin and Berichrom® Antithrombin III (A)

The *Innovance*® Antithrombin assay was well correlated to the Berichrom® Antithrombin III (A) assay as demonstrated by a Pearson correlation coefficient of 0.96, a slope of 0.98 and an intercept of 1.56 % (n=120, Figure 2). For the correlation analysis, samples with FIIa and FXa inhibitors were excluded (see Results and Discussion, point 3).

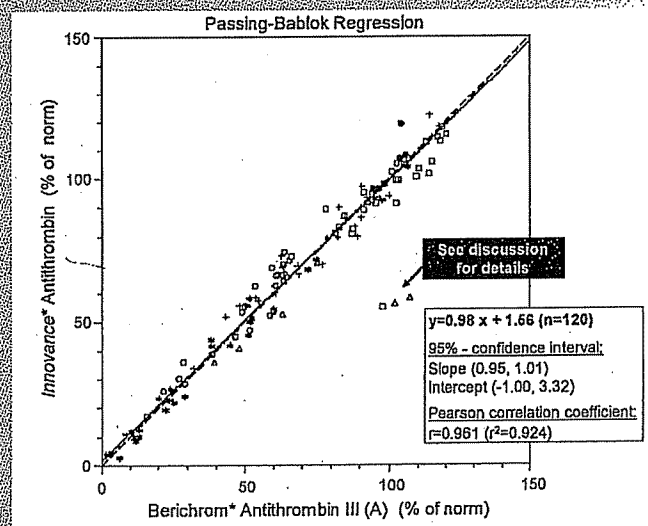


Figure 2: Comparison between *Innovance*® Antithrombin

Design and Methods:

Samples were measured from patients with hereditary AT deficiency (n=7), patients with acquired AT deficiency (n=22), patients with normal AT activity (n=30) and patients receiving heparin (n=25) or Lepirudin (n=6). In addition, diluted plasma samples (n=30), plasma spiked with AT concentrate (n=6, target concentration: 80 – 120 %) and plasma spiked with Fondaparinux (n=12, target concentration: 1.2-1.8 µg/mL) were tested. The precision of the new assay was evaluated by testing three commercial controls and two plasma pools on 20 days (two runs per day, two measurements per run). All measurements were performed on Sysmex® CA-1500 System.

All patients with hereditary AT deficiency were correctly identified with *Innovance*® Antithrombin whereas 2 out of 7 patients had normal AT activity with Berichrom® Antithrombin III (A) (Figure 2). Both of these patients carry the same Pro73Leu mutation, which impairs interaction of AT with heparin. Interestingly, the two patients were identified as pathological if Berichrom® Antithrombin III (A) was performed with decreased incubation time of the diluted samples with thrombin. The increase of sensitivity for the detection of type II HBS (heparin binding site) defects by decreasing the incubation time has been demonstrated earlier[2,4].

One patient demonstrated normal AT activity with Berichrom® Antithrombin III (A) but deficient AT activity using *Innovance*® Antithrombin (97.9 and 55.2 % respectively). This sample was pathological (71 %) using the Berichrom® Antithrombin III (A) assay with decreased incubation time. Two dimensional electrophoresis with heparin showed a normal fast moving peak and an abnormal slow moving peak, indicating heterozygous type II HBS deficiency.

3. Influence of Factor Xa and Factor IIa inhibitors on *Innovance*® Antithrombin and Berichrom® Antithrombin III (A) assays

As depicted in Figure 3, samples from patients under therapy with the thrombin inhibitor Lepirudin demonstrated slightly higher values in the FIIa-based Berichrom® Antithrombin III (A) assay compared to the FXa-based *Innovance*® Antithrombin assay. Fondaparinux (FXa inhibitor) spiked samples showed 10-50 % higher values using *Innovance*® Antithrombin assay compared to the Berichrom® Antithrombin III (A) assay.

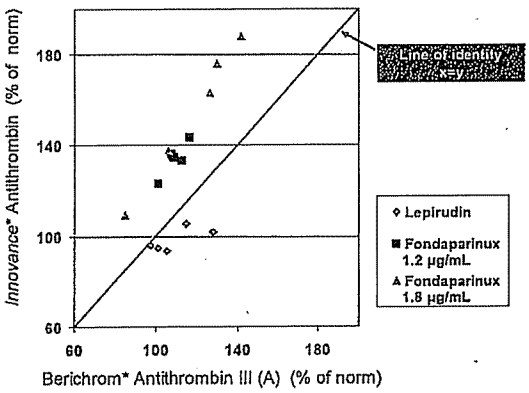


Figure 3: Influence of Lepirudin and Fondaparinux on AT assays

Conclusions:

***Innovance*® Antithrombin demonstrated high precision and excellent comparability to the Berichrom® Antithrombin III (A) method. *Innovance*® Antithrombin had higher sensitivity to a type IIb AT deficiency (Pro73Leu mutation affecting heparin binding site) than the FIIa-based method. The *Innovance*® Antithrombin assay is easy to perform in particular because of the ready-to-use reagents.**

References:

[1] Bohrer J, von Pape KW, Blaurock M. Thrombin-based antithrombin assays show overestimation of antithrombin III activity in patients on heparin therapy due to heparin cofactor II influence. Thromb Haemost 1994; 71: 280-3
[2] Kristensen SR, Rasnzssen B, Pedersen S, Bathum L. Detecting antithrombin deficiency may [illegible]